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# New data on and the easternmost record of the Javan endemic Pearly Tree Frog, *Nyctixalus margaritifer* Boulenger, 1882 (Anura, Rhacophoridae)

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**Abstract.** New locality records and a range extension of the Javan endemic Pearly Tree Frog, *Nyctixalus margaritifer* Boulenger, 1882 are reported from Central Java and East Java. Our new data include the easternmost record in Java, a record from the eastern edge of Bromo Tengger Semeru National Park, which is approximately 142 km west of the species' type locality in Gunung Willis in East Java. This tree-frog species inhabits undisturbed rainforest with a dense canopy and vegetation at elevations up to 1,200 m a.s.l. Based on a morphometric analysis of newly collected specimens, we found observable differences in several morphological characters. However, more specimens are needed to confirm the taxonomic status of the rare, endemic, and poorly known *N. margaritifer.*

**Keywords.** Amphibian, distribution, Indonesia, molecular phylogeny

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## Introduction

The tree-frog genus *Nyctixalus* Boulenger, 1882 (Anura, Rhacophoridae) includes *Nyctixalus pictus* (Peters 1871) from Sundaland and Palawan in the Philippines (Smith 1931; Iskandar 1998; Chan-ard 2003); *N. spinosus* (Taylor 1920) from southern Philippine archipelagos (Sanguila et al. 2016); and *N. margaritifer* Boulenger, 1882 (Fig. 1), which is endemic to Java Island (Iskandar 1998). Due to the rarity of *N. margaritifer*, there is a lack of information on the natural history, distribution, and population status of this species.

*Nyctixalus margaritifer* is well documented in West Java, where it is known from Gunung Gede near Tjibodas (Barbour 1912), Gunung Gede Pangrango National Park (Iskandar 1998), Gunung Halimun Salak National Park (Mumpuni 2001; Kurniati 2005), Bandung and Lebak Regencies (IUCN SSC Amphibian Specialist Group 2018), and Telaga Warna Nature Reserve (Setiawan et al. 2019). In Central Java, it was only known

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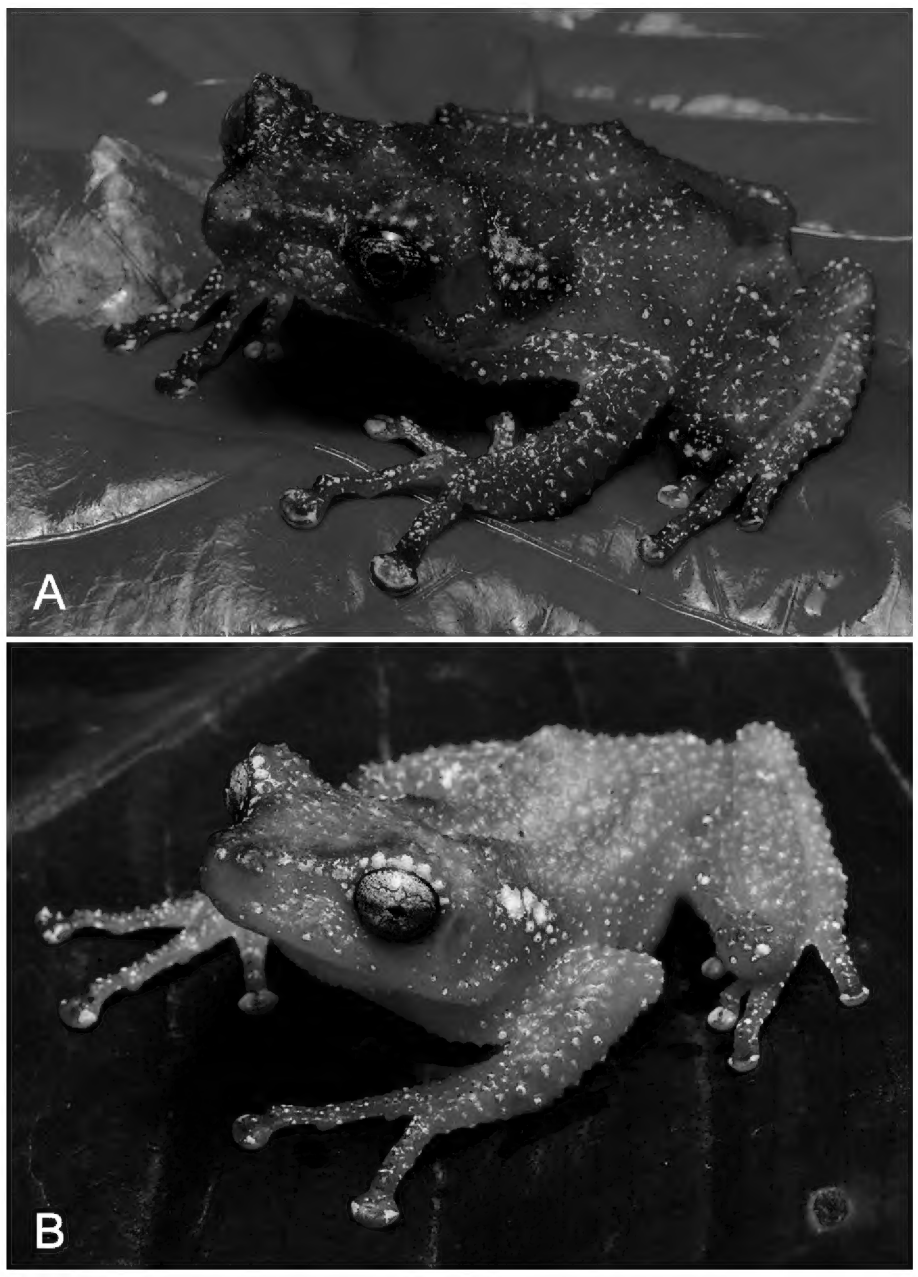


**Figure 1.** In life photos (ex-situ) of adult females *Nyctixalus margaritifer*. **A.** NK 2114 from Resort PTN Senduro. **B.** NK 2122 from Resort PTN Ranu Darungan, Bromo Tengger Semeru National Park (BTSNP), East Java, Indonesia. Photos: L. Septiadi.

from Gunung Ungaran and Gunung Merapi National Park (IUCN SSC Amphibian Specialist Group 2018). However, *N. margaritifer* has not been reported from East Java, but surveys of the herpetofauna of East Java has been very minimal (Kurniawan et al. 2021; Kusrini et al. 2021). *Nyctixalus margaritifer* was first described on Gunung Wilis (in English "gunung" is a mountain) in East Java (Smith 1931; Dubois 1981) and only rediscovered after 135 years at Sigogor Nature Reserve, which is near the type locality (Priambodo et al. 2021). With the increasing frequency and intensity of exploration in recent years in East Java (Septiadi et al. 2023), it is possible that the distribution of this species could be expanded to other locations.

We discovered individuals of Pearly Tree Frog, *N. margaritifer*, during our exploration at the Bromo Tengger Semeru National Park (BTSNP) in East Java, and additional individuals in Banyumas Regency and Purbalingga City (Central Java). Our record of this species from the eastern edge of BTSNP is the easternmost record of *N. margaritifer* in Java.

## Methods

Field surveys were conducted in Resort PTN Senduro and Resort PTN Darungan, BTSNP, East Java, Indonesia during March–April 2022. These surveys found two specimens. We also made observations in Limpakuwus Village, Banyumas Regency, and Serang Pratin Village, Purbalingga City, both in Central Java, Indonesia during April 2022, which resulted in four collected specimens. The specimens were captured by hand and immediately transferred to the laboratory for observation. We took notes on habitat, elevation, and coordinates using a Garmin eTrex 10 GPS receiver using the WGS84 datum. A Nikon D5200 camera was used to take a series of photographs of the live specimens (ex-situ). Six specimens were euthanized with 7.5% benzocaine, fixed in 10% formalin for 24 h, stored in 70% ethanol, and deposited at the Laboratory of Animal Diversity and Ecology Collection, Biology Department, Universitas Brawijaya, Indonesia (NK). For further molecular analyses, a tissue sample was collected in 95% ethanol.

In our morphological analyses, we compared specimens of *N. margaritifer* from various populations in Java. These include our newly collected specimens from East Java ($n$ = 2; NK 2114 and NK 2122), our newly collected specimens from Central Java (n = 4; NK 2118-19 and NK 2123-24), previously collected specimens from West Java ($n$ = 4; RMB 2775/6749, HK 241/10.917, RMB 2419/6686, and JAM 3130/6751), and data provided by Priambodo et al. (2021) on a specimen from East Java ($n$ = 1, female). Of the 10 adult specimens we analysed, two were male and eight were female. Specimens studied by us are available in the Laboratory of Animal Diversity and Ecology Collection, Biology Department, Universitas Brawijaya, Indonesia (NK), and Museum Zoologicum Bogoriense, BRIN-Cibinong, Indonesia (MZB); see Appendix, Table A1 for detailed information. The geographical distribution of *N. margaritifer* was determined from literature records (including Priambodo et al. 2021), specimens in collections, and our new data. We mapped the geographic distribution of *N. margaritifer* using QGIS v. 3.8.1.

Morphometric data were recorded for the following 24 characters as defined by Watters et al. (2016) using vernier calipers (accuracy 0.05 mm): SVL = snout to vent length; A–G = axilla to groin, distance from posterior base of forelimb at its emergence from body to anterior base of hind limb at its emergence from body; HL = head length from rear of lower jaw to tip of snout; HW = head width at greatest cranial width; IN = internarial distance between nostrils; SL = distance of tip of snout to eye; IOD = interorbital distance; UEW = upper eyelid width, greatest width of upper eyelids; EL = horizontal diameter of eye; TD = horizontal diameter of tympanum; FLL = length of forelimb from tip of disk of third finger to axilla; HAL = hand length, from base of outer palmar tubercle to the tip of third finger; LAL = forearm length, from elbow to base of outer palmar tubercle; FAL = upper arm length, from body to elbow; 3FL = third finger length; FTB = maximal diameter of third finger disk; HLL = length of hindlimb from tip of fourth toe disk to groin; TFOL = length of hindlimb from tip of fourth toe to posterior edge of tibia; FOL = length of metatarsal tubercle to

the tip of 4th toe; TL = tibia length; THIGHL = femur length; IMTL = length of internal metatarsal tubercle; 4TL = fourth toe length; and HTD = diameter of fourth toe tip, greatest diameter of disk on fourth toe. Webbing formula follows Savage and Heyer (1997). Sex was determined by the presence of nuptial pads and gonadal inspection.

To anticipate bias from ontogenetic variation (Chan and Grismer 2021), we conducted an allometric body-size correction using the GroupStruct R package (Chan and Grismer 2022). We separated the analysis by sex. However, as the sample sizes for adult male specimens in Java ($n$ = 2) and adult female specimens from Central Java ($n$ = 2) were too small, we only included adult females ($n$ = 7) from East Java and West Java populations in the principal component analysis (PCA); see Appendix, Table A1. In addition, we incorporated 14 morphometric characters (of 24 morphometric characters measured) following Priambodo et al. (2021). Prior to the PCA, we performed a univariate analysis to check the normality assumption, homogeneity of variance, and significant differences ($t$-test) among characters across populations, following the defensible framework by Chan and Grismer (2021). By using the PCA to find the best low-dimensional representation of variation in the data, we were able to investigate the morphological variation of the detectable group or population. The specimens were compared between sexes. The coloration (dorsal, ventral, and lateral sides), skin texture, and the presence of skin folds, webbing of limbs, and vomerine teeth were recorded.

Mitochondrial DNA of 12S rRNA, $tRNA^{val}$, and 16S rRNA genes was extracted from tissue samples from NK 2114 and NK 2122; we used QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. We amplified and sequenced (Sanger sequencing) the genes using primers L2 LX12SN (5′–TACACACCGGCC GTCA–3′), L2 LX16S1R (5′–GACCTGGATTACTCCG GTCTGAACTC–3′), L1 16S2000H (5′–GTGATTAYGC TACCTTTGCACGGT–3′), and L1 12SAL (5′–AAACT GGGATTAGATACCCCACTAT–3′) following Zhang et al. (2013). Our derived sequences were assembled and edited using BIOEDIT v. 7 (Hall 2011) and deposited in GenBank (OQ555086 and OQ555087). Four newly collected samples from Central Java were not successfully sequenced and were thus not analyzed. We aligned the two successful sequences along with eight sequences of *N. margaritifer* from Central Java and West Java populations ($n$ = 2), *N. pictus* ($n$ = 4), *N. spinosa* ($n$ = 2), and one outgroup, *Buergeria otai*, from GenBank using ClustalW in MEGA v. 7 (Kumar et al. 2016) with default parameters and adjusted manually whenever necessary; the outgroup was determined following Poyarkov et al. (2015); see Table 1 for GenBank accession numbers, voucher ID, and references. An uncorrected $p$-distance was calculated in MEGA v. 7 (Kumar et al. 2016) for the 16S rRNA gene only. Nucleotide substitution model was estimated using JMODELTEST2 (Darriba et al. 2012). The GTR was considered as the best-fit substitution model for maximum likelihood (ML) and Bayesian inference (BI) of the concatenated 12S rRNA, $tRNA^{val}$, and 16S rRNA gene alignment. Phylogenetic inferences based on the ML were estimated using IQ-TREE (Nguyen et al. 2015a) with 1,000 ultrafast bootstrap replicates (UFBoot) for assessing branch supports (Hoang et al. 2018). MRBAYES v. 3.2.7a (Ronquist et al. 2012), with Markov Chains Monte Carlo (MCMC) for 1,000,000 generations, was used in the BI for assessing branch supports (Bayesian posterior probability; BPP). We consider UFBoot ≥ 95 and BPP ≥ 0.95 to be well supported (Kurniawan et al. 2023); both were analyzed through the CIPRES Science Gateway (Miller et al. 2012). We visualized the trees using FIGTREE v. 1.3.1. (Rambaut 2009).

**Table 1.** Sample used in the molecular analyses with information on voucher ID, locality, GenBank accession, and reference. An asterisk (*) indicates the species name was modified from that provided in the GenBank accession at the time of download due to taxonomic revision.

| Species | Voucher ID | Locality | GenBank accession | Reference |
|---|---|---|---|---|
| *Buergeria otai** | UMFS 5821 | Taiwan: I-Lan | DQ283055 | Frost et al. 2006 |
| *Nyctixalus pictus* | FMNH 231095 | Malaysia: Sabah: Lahad Datu | DQ283133 | Frost et al. 2006 |
| *Nyctixalus pictus* | FMNH 231094–2 | Malaysia: Sabah: Lahad Datu | AF458135 | Wilkinson et al. 2002 |
| *Nyctixalus pictus* | KUHE 53517 | Malaysia: Sarawak | LC012863 | Nguyen et al. 2015b |
| *Nyctixalus pictus* | MVZ 239460 | Indonesia: Sumatra: Bengkulu | GQ204783; GQ204732 | Meegaskumbura et al. 2010 |
| *Nyctixalus margaritifer* | TNHC JAM 3030 | Indonesia: West Java: Gn. Gede Pangrango | EU178094 | Biju et al. 2008 |
| *Nyctixalus margaritifer* | KUHE 26135 | Indonesia: Central Java: Gn. Ungaran | LC012864 | Nguyen et al. 2015b |
| *Nyctixalus margaritifer* | NK 2114 | Indonesia: East Java: Resort PTN Senduro, BTSNP | OQ555086 | this study |
| *Nyctixalus margaritifer* | NK 2122 | Indonesia: East Java: Resort PTN Darungan, BTSNP | OQ555087 | this study |
| *Nyctixalus spinosus* | ACD 1043 | Phillipines: Mindanao | DQ283114 | Frost et al. 2006 |
| *Nyctixalus spinosus* | Pet trade | Phillipines: Mindanao | KT461916 | Poyarkov et al. 2015 |

## Results

***Nyctixalus margaritifer* Boulenger, 1882**

Figures 1, 2

**New records.** INDONESIA – **East Java Province** • Bromo Tengger Semeru National Park, Resort PTN Senduro; 08°03′09″S, 113°01′51″E; 1,103 m alt.; 27.III.2022; Dava R. Azizi leg.; primary forest habitat near road; 1 ♀, NK 2114 • same locality, Resort PTN Ranu Darungan; 08°11′23″S, 112°55′35″E; 849 m alt.; 20.IV.2022; Berry F. Hanifa leg.; primary forest habitat near lake; 1 ♀, NK 2122 – **Central Java Province** • Purbalingga City, Serang Pratin Village; 07°14′36″S, 109°17′18″E; 1,142 m alt.; 19.IV.2022; Miftah Farid leg.; secondary forest habitat; 1 ♀, NK 2118; 1 ♀, NK 2119 • Banyumas Regency, Purwokerto District, Limpakuwus Village; 07°17′52″S, 109°14′59″E; 835 m alt.; 26.IV.2022; Miftah Farid leg.; secondary forest habitat near hiking trail; 1 ♂, NK 2123; 1 ♂, NK 2124.

**Identification.** *Nyctixalus margaritifer* is endemic to Java (Iskandar 1998). Its closest congeners are *N. pictus* in Sumatra, Borneo, Malay Peninsula, Peninsular

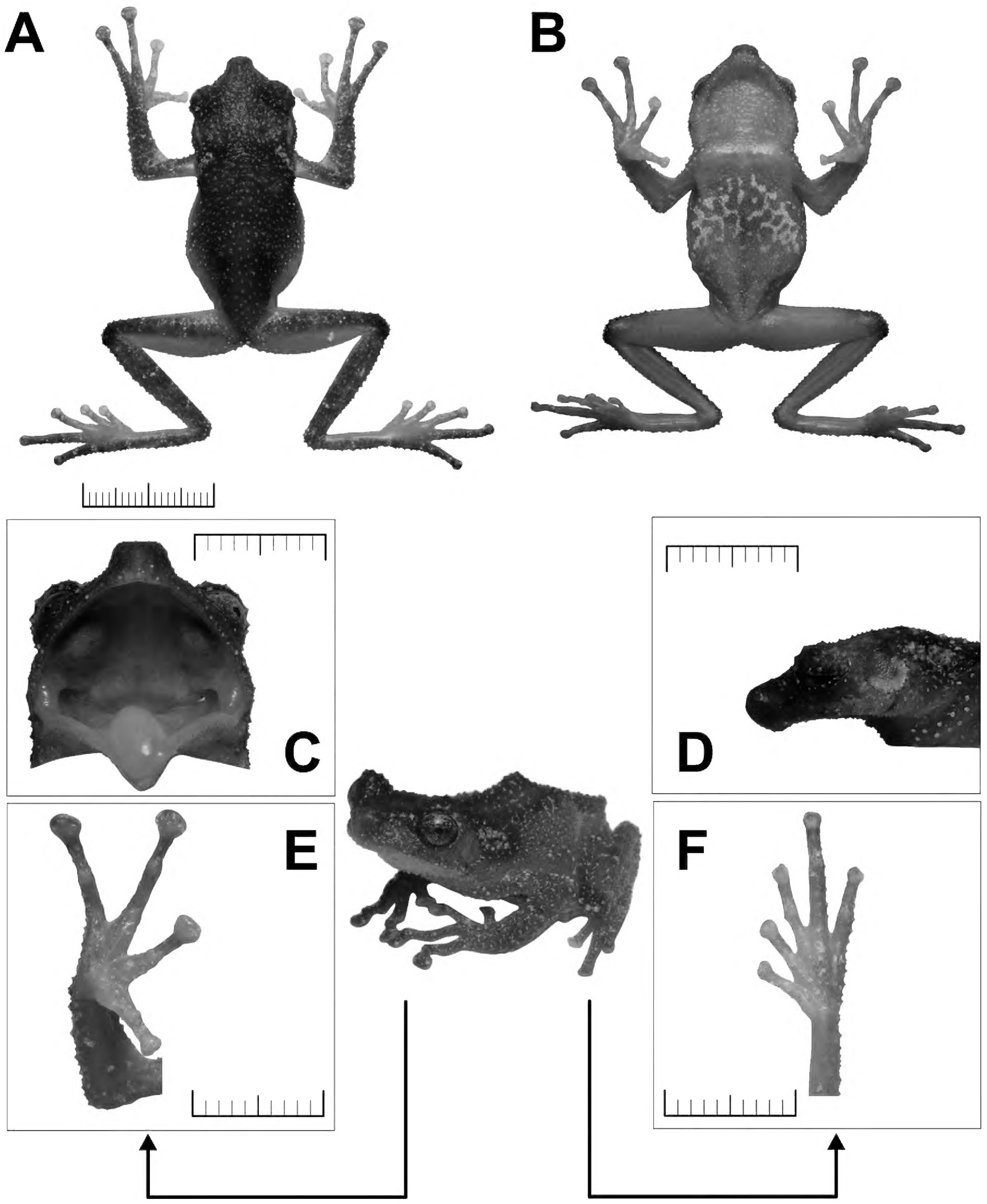


**Figure 2.** Freshly euthanized adult female *Nyctixalus margaritifer* (NK 2114) from Resort PTN Senduro, Bromo Tengger Semeru National Park (BTSNP), East Java, Indonesia. **A.** Dorsal. **B.** Ventral. **C.** Upper mouth part. **D.** Lateral head. **E.** Ventral right hand. **F.** Ventral right foot. Scale bars: A, B = 20 mm; C–F = 10 mm. Photos: L. Septiadi.

Thailand, Peninsular Myanmar, Vietnam, and Palawan Island of the Philippines (Smith 1931; Iskandar 1998; Chan-ard 2003) and *N. spinosus* in the Philippines (Mindanao, Leyte, Bohol, and Basilan islands) (Sanguila et al. 2016).

Morphological characters of these specimens (*n* = 6; NK 2114, NK 2118-19, and NK 2122-24) matched well with the diagnostic key of *N. margaritifer* provided by Brown and Alcala (1994) and Boulenger (1882), which includes: (1) cranial and supratympanic crests absent (vs. present in *N. spinosus*), (2) dorsal surfaces covered by several pointed spicules and small pearl-like tubercles (vs. weak spicules and smooth tubercles in *N. pictus*), (3) throat and posterior thighs with coarse granules (vs. smooth granules in *N. pictus*), and (4) webbing reaches basal subarticular tubercle of fourth toe (vs. reaches penultimate subarticular tubercle in *N. pictus*).

**Phylogeny and genetic distance.** Aligned sequence matrix of 12S rRNA, $tRNA^{val}$, and 16S rRNA (1,924 bp) comprises 489 parsimony-informative, 216 singleton sites, and 1,214 conserved sites. Phylogenetic trees of both ML and BI demonstrated identical topologies and well-supported nodes (Fig. 3) and suggest that these specimens were nested in the genus *Nyctixalus* and form monophyletic clade with *N. margaritifer*. The newly collected specimens from East Java were sister to *N. margaritifer* from Central and West Java, with their monophyly being supported. Among *Nyctixalus* species, the uncorrected *p*-distance of the 16S rRNA gene ranged from 0.0 to 11.4%, whereas the genetic distance between *N. margaritifer* among populations from Central Java, West Java, and East Java were as large as 0.7–1.7% (Table 2). Therefore, we conclude that these newly collected specimens are *N. margaritifer*.

**Description.** A medium-sized rhacophorid (*n* = 6; NK 2114, NK 2118-19, and NK 2122-24); body slender, dorsoventrally compressed. Skin below posterior axial of left hindlimb laterally dissected. Head wider than longer (HL/HW = 0.63–0.86), strongly depressed; snout rather long (SL/HL = 0.39–0.47), prominent, tip obliquely truncate backwards and downwards; nostril ovoid, protuberant, oriented dorsolaterally, closer to tip of snout than to eye; canthus rostralis angular; loreal region concave; interorbital space concave, smaller to broader than upper eyelid (EUW/IOD = 0.61–1.26); eye large (EL/HL = 0.36–0.48), eye diameter similar to snout length (EL/SL = 0.87–1.01), protuberant in dorsal view; pupil diamond-shaped when contracted, small and horizontally rounded when dilated; tympanum distinct, rounded, slightly larger than eye diameter (TD/EL = 0.65–0.92); vomerine teeth absent; choanae oval; pinneal eye absent; tongue wide, spatulate, free at posterior end, attached anteriorly (Fig. 2A–D).

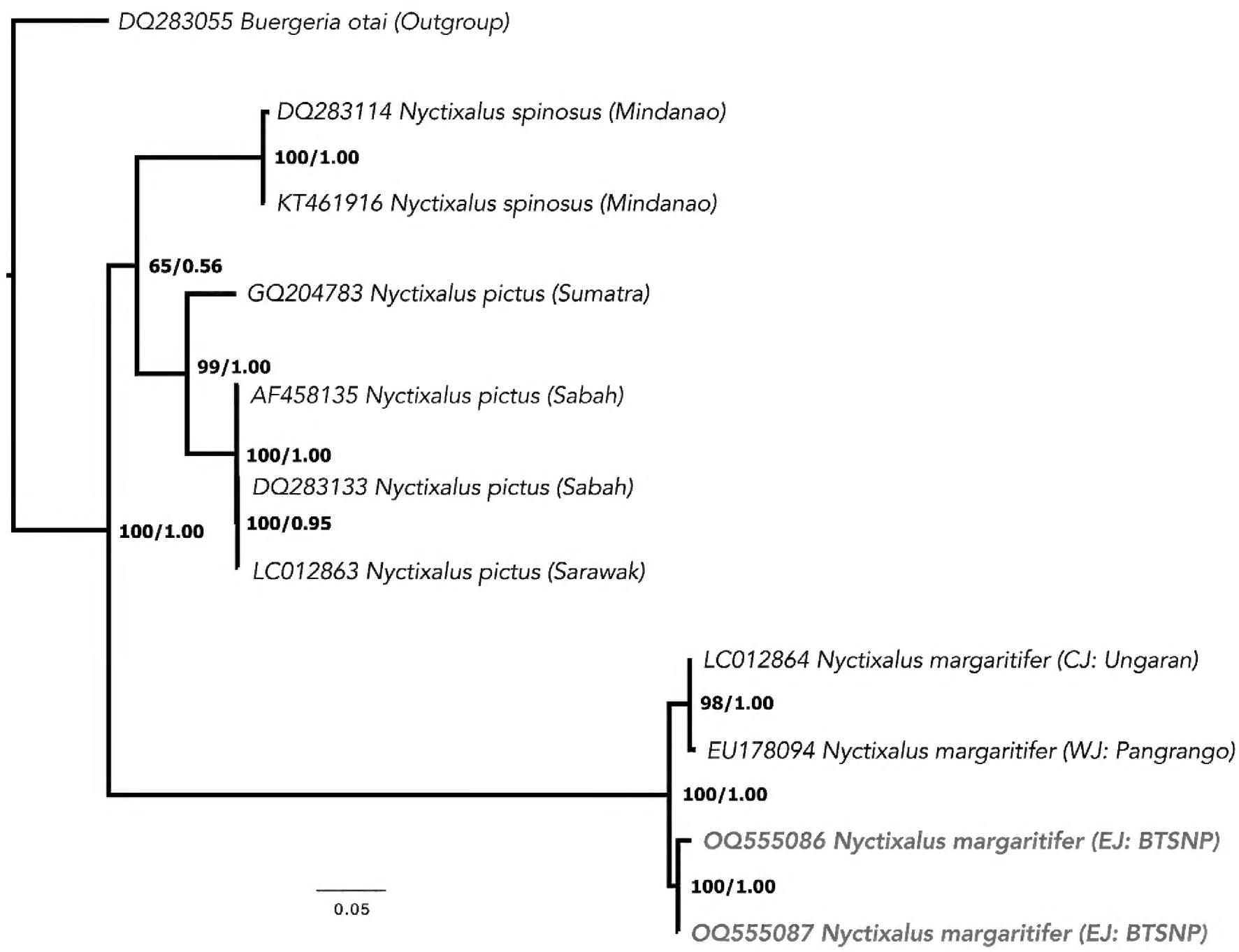


**Figure 3.** Bayesian-inference (BI) tree of concatenated 12S rRNA, $tRNA^{val}$, and 16S rRNA alignment showing inferred relationship among *Nyctixalus* spp. The bootstrap supports (UFBoot) and Bayesian posterior probability values are shown at the internal branch.

**Table 2.** Uncorrected *p*-distances (in %) for 16S rRNA gene fragment (598 bp) among *Nyctixalus* spp. taxa compared in this study. We excluded two sequences from this analysis (GQ204783 and KT461916) since these sequences either partially contained short fragments of 16S rRNA or only contained other fragments (12S rRNA and tRNA$^{val}$).

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 DQ283114 *Nyctixalus spinosus* (Mindanao) | 0.00 | | | | | | | |
| 2 DQ283133 *N. pictus* (Sabah) | 9.73 | 0.00 | | | | | | |
| 3 AF458135 *N. pictus* (Sabah) | 9.73 | 0.00 | 0.00 | | | | | |
| 4 LC012863 *N. pictus* (Sarawak) | 9.73 | 0.00 | 0.00 | 0.00 | | | | |
| 5 EU178094 *N. margaritifer* (West Java: Pangrango) | 10.74 | 11.14 | 11.14 | 11.14 | 0.00 | | | |
| 6 LC012864 *N. margaritifer* (Central Java: Ungaran) | 9.88 | 10.26 | 10.26 | 10.26 | 0.72 | 0.00 | | |
| 7 OQ555086 *N. margaritifer* (East Java: BTSNP) | 10.46 | 11.41 | 11.41 | 11.41 | 1.70 | 0.97 | 0.00 | |
| 8 OQ555087 *N. margaritifer* (East Java: BTSNP) | 10.46 | 11.41 | 11.41 | 11.41 | 1.70 | 0.97 | 0.00 | 0.00 |

Forelimbs slender, relative fingers lengths: I < II < IV < III; tip of fingers with well-developed disks, distinctly circummarginal grooves; largest disk on third finger; disks slightly trapezoid, less than tympanum diameter (FTB/TD = 0.49–0.72); fingers slightly webbed, almost reaching basal subarticular tubercle, with extending narrow fringe along the sides; subarticular tubercles developed, finger subarticular formula: I (2), II (2), III (3), IV (3); distinct metacarpal tubercles (Fig. 2E).

Hindlimbs slender, relatively long, tibio–tarsal articulation reach beyond tip of snout, tibia over half of snout–vent length (TL/SVL = 0.51–0.52); dermal thickening on femur–tibia fold on outer aspect present; toes slightly webbed, almost reaching basal subarticular tubercle, with extending narrow fringe along the sides, varies toe webbing formula; tip of toes with less-developed disks, distinctly circummarginal grooves; disks slightly trapezoid, smaller than fingers (HTD/FTB = 0.48–0.63), largest disk on fourth toe; relative toes lengths I < II < III < V < IV; subarticular tubercles moderately developed, toe subarticular formula: I (2), II (2), III (3), IV (4), V (3); very indistinct inner metatarsal tubercle present (Fig. 2F).

Skin texture on dorsal head, lateral head, body, forelimbs, and hindlimbs with blunt, pointed, whitish spicules and very small, pearl-like, scattered tubercles; skin co-ossified to skull; tympanum surrounded by very small tubercles, granulate at center; upper eyelid tubercular; supratympanic crest absent; throat, belly, and posterior thighs with coarse granules (Fig. 2).

Dorsal surface light to dark brown, with yellowish or whitish spot on each scapula, scattered whitish spots forming transverse, band-like shape on dorsal surface of forelimbs, fingers, hindlimbs, and toes; whitish spots on tubercles of canthus rostralis, and upper and lower eyelids; tympanum uniformly dark brown; ventral surface marbled with light brown; belly with irregular black and white streaks (Figs. 1, 2).

**Morphometric analysis and variations.** Based on the PCA analysis ($n$ = 7; see Appendix, Table A1), PC1 had the eigenvalue of 6.64 indicating that most variation (47%) is contributed by the first axis (PC1). Along the PC1, the West Java population is separated from the East Java population (Fig. 4). The second axis (PC2) had the eigenvalue of 3.66 indicating that the variation (26%) also contributed to further separate West Java and East Java populations. PCA loading for PC1 ranged from –0.37 to 0.38, with heavier loadings on LAL, THIGHL, and FOL (see Appendix, Table A2). The *t*-test showed that the populations of West Java and East Java are significantly different ($p < 0.05$) in three assessed characters: HL, SL, and LAL. Although there were observable differences between the populations, the small number of specimens in our PCA analysis is

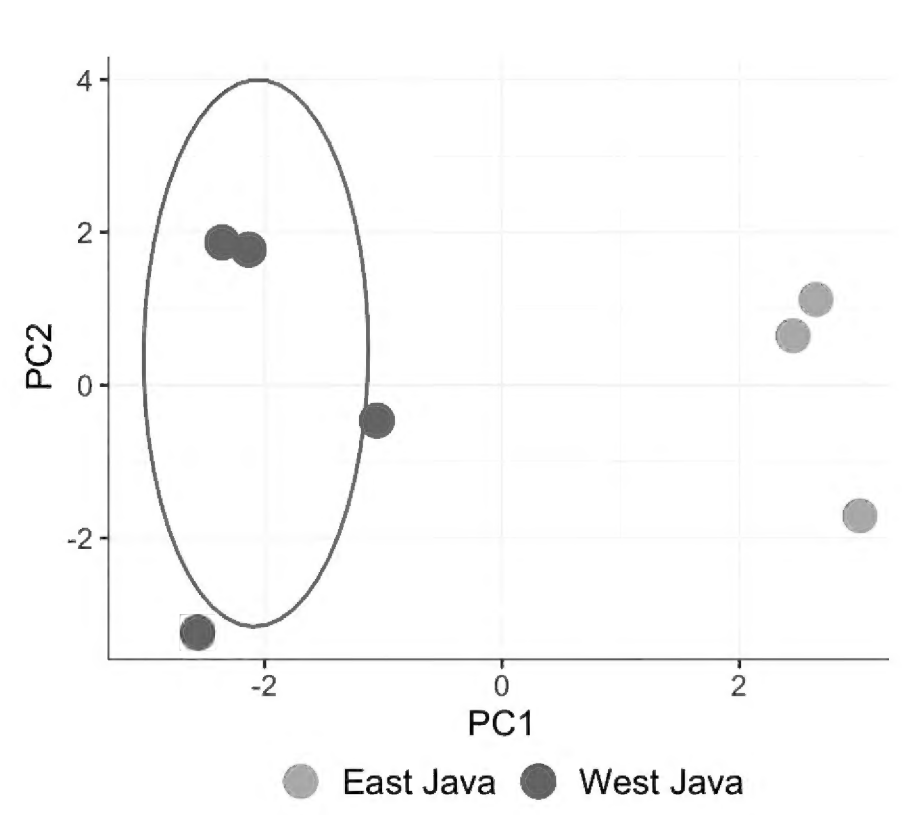


**Figure 4.** Plot of first-two principal components of adult female *Nyctixalus margaritifer* grouped by East Java and West Java population, using 14 allometric body-size correction characters incorporating BP UM 0011 measurements by Priambodo et al. (2021).

**Table 3.** Measurements of 24 morphometric characters (in mm) among sexes of *Nyctixalus margaritifer*. See Methods for abbreviations.

| Characters | Adult male (*n* = 2) | | Adult female (*n* = 8) | |
|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range |
| SVL | 44.8 ± 0.8 | 44.2–45.4 | 44.2 ± 2.2 | 41.5–48.4 |
| A–G | 19.0 ± 1.9 | 17.6–20.4 | 20.2 ± 2.5 | 17.0–23.6 |
| HL | 12.4 ± 0.7 | 11.9–12.9 | 14.0 ± 2.5 | 10.1–17.0 |
| HW | 16.7 ± 0.0 | 16.7–16.7 | 16.2 ± 0.6 | 15.3–17.1 |
| IN | 3.0 ± 0.2 | 2.9–3.2 | 2.6 ± 0.5 | 1.6–3.3 |
| SL | 5.3 ± 0.2 | 5.2–5.4 | 5.9 ± 0.8 | 4.7–7.2 |
| IOD | 5.9 ± 0.8 | 5.3–6.5 | 5.9 ± 0.8 | 4.0–6.7 |
| EUW | 4.5 ± 0.1 | 4.5–4.5 | 4.2 ± 0.6 | 3.6–5.1 |
| EL | 5.1 ± 0.2 | 5.0–5.2 | 4.6 ± 0.5 | 3.8–5.2 |
| TD | 4.1 ± 0.2 | 4.0–4.2 | 3.9 ± 0.4 | 3.4–4.4 |
| FLL | 31.6 ± 1.5 | 30.5–32.7 | 34.0 ± 3.1 | 28.6–39.0 |
| HAL | 15.1 ± 0.1 | 15.0–15.2 | 15.0 ± 0.8 | 13.9–16.4 |
| LAL | 12.2 ± 1.0 | 11.5–12.9 | 10.0 ± 1.5 | 8.1–12.4 |
| FAL | 8.9 ± 1.2 | 8.0–9.7 | 9.5 ± 0.5 | 8.8–10.3 |
| 3FL | 12.5 ± 0.4 | 12.2–12.8 | 12.2 ± 0.8 | 11.0–13.4 |
| FTB | 2.7 ± 0.3 | 2.5–2.9 | 2.0 ± 0.5 | 1.2–2.6 |
| HLL | 69.5 ± 2.1 | 68.0–71.0 | 70.8 ± 3.2 | 65.5–74.5 |
| TFOL | 30.3 ± 1.0 | 29.6–31.1 | 29.2 ± 2.3 | 26.0–32.9 |
| FOL | 17.8 ± 0.3 | 17.6–18.0 | 17.0 ± 1.7 | 14.2–19.8 |
| TL | 23.1 ± 0.1 | 23.0–23.2 | 23.1 ± 1.2 | 21.4–25.5 |
| THIGHL | 22.1 ± 0.4 | 21.8–22.4 | 21.4 ± 0.8 | 20.3–22.9 |
| 4TL | 12.8 ± 0.7 | 12.3–13.3 | 13.1 ± 1.8 | 10.6–15.8 |
| IMTL | 2.5 ± 0.4 | 2.3–2.8 | 2.2 ± 0.5 | 1.6–3.4 |
| HTD | 1.6 ± 0.2 | 1.5–1.7 | 1.2 ± 0.3 | 0.9–1.7 |

insufficient to conclude a distinction between West Java and East Java populations.

Apart from visible secondary sex characters, sexual dimorphism was observed in seven morphometric characters of adult males and females ($n$ = 2 for ♂; $n$ = 8 for ♀; see Appendix, Table A1). A–G in adult females (mean ± SD = 20.2 ± 2.5) is slightly longer than in adult males (19.0 ± 1.9), HL in adult females (14.0 ± 2.5) is longer than in adult males (12.4 ± 0.7), HW in adult females (16.2 ± 0.6) is slightly smaller than in adult males (16.7 ± 0.0), SL in adult females (5.9 ± 0.8) is slightly longer than in adult males (5.3 ± 0.2), FLL in adult females (34.03 ± 3.1) is longer than in adult males (31.6 ± 1.5), LAL in adult females (10.0 ± 1.5) is shorter than in adult males (12.2 ± 1.0), and HLL in adult females (70.8 ± 3.2) is slightly longer than in adult males (69.5 ± 2.1). The comparison of 24 morphometric characters between the sexes is shown in Table 3. Toe webbing overlaps between sexes, as shown in Table 4.

Specimens from BTSNP ($n$ = 2; NK 2114 and NK 2122) lack the whitish spot forming a band-like shape on the dorsal surface of the forelimbs, fingers, hindlimbs, and toes, whereas specimens from East Java ($n$ = 1; BP UM 0011) and Central Java ($n$ = 4; NK 2118-19, and NK 2123-24) have a prominent whitish spot.

**Table 4.** Toe webbing variations of *Nyctixalus margaritifer* between sexes. See Appendix, Table A1 for details on localities.

| Voucher ID | Sex | Toe webbing formula |
|---|---|---|
| NK 2123 | Adult male | I 2 - 2½ II 2 - 3½ III 3 - 4 IV 4 - 2½ V |
| NK 2124 | | I 2 - 2½ II 2 - 3½ III 2½ - 4 IV 4 - 2½ V |
| NK 2118 | Adult female | I 2 - 2½ II 2 - 3½ III 2½ - 4 IV 4 - 3 V |
| NK 2119 | | I 2 - 2½ II 2 - 3 III 2½ - 3½ IV 4 - 3 V |
| NK 2114 | | I 2 - 2 II 2 - 3½ III 2½ - 3½ IV 3½ - 2½ V |
| NK 2122 | | I 2 - 2½ II 2 - 3½ III 2½ - 4 IV 4 - 2½ V |
| BP UM 0011 | | I 2 - 2 II 2 - 3½ III 2½ - 4 IV 4 - 3 V |
| RMB 2775/6749 | | I 2 - 2 II 2 - 3 III 3 - 4 IV 4 - 2½ V |
| HK 241/10.917 | | I 2 - 2 II 2 - 3 III 3 - 4 IV 4 - 2½ V |
| RMB 2419/6686 | | I 2 - 2 II 2 - 3 III 3 - 4 IV 4 - 2½ V |
| JAM 3130/6751 | | I 2 - 2 II 2 - 3 III 3 - 4 IV 4 - 2½ V |

**Habitat and natural history.** The first individual (NK 2114) was found near the road, about 10 m near the resort of PTN Senduro, BTSNP, among deep forest and shrub vegetation. It was on a leaf of *Amischotolype mollisima* (Blume) Hassk., approximately 50 cm above the ground (Fig. 5A), during the rainy season (air temperature 23 °C, air humidity 78%); there was parasitic worm

**Figure 5.** Habitat at collection site of two specimens of *Nyctixalus margaritifer*. **A.** NK 2114 from Resort PTN Senduro. **B.** NK 2122 from Resort PTN Ranu Darungan, Bromo Tengger Semeru National Park (BTSNP), East Java, Indonesia. Photos: A.M.S.P. Soeprijadi.

on right eye of NK 2114 (Fig. 1A). The second individual (NK 2122) was collected from a shrub, about 25 cm above the ground on the hill area of the Dutch Loji relic, approximately 100 m from the Ranu Linggo Rekisi lake (Fig. 5B), during the rainy season (air temperature 21°C, air humidity 76%). The third to sixth individuals (NK 2118-19, and NK 2123-24) from Banyumas Regency and Purbalingga City shared generally the same habitat as the individuals from the East Java population, but were found clustered and collected in dense shrubs, far from the nearest village.

In the hill area of the Dutch Loji relic of BTNSP, the calls of males were heard in the high tree canopy formed by bird's nest fern (*Asplenium* sp.) in the evening (18:00–20:00), just following a light rain when the forest floor was still wet. Several anuran species were found living sympatrically with two individuals (NK 2114 and NK 2122), including *Philautus aurifasciatus* (Schlegel, 1837), *Philautus* sp., *Odorrana hosii* (Boulenger, 1891), *Hylarana chalconota* (Schlegel, 1837), *Microhyla achatina* Tschudi, 1838, and *Microhyla* sp. The population size, habitat requirement, and reproduction mode were not observed. After direct contact, one individual (NK 2122) exhibited a defensive posture by curling its body into a ball, arching its head, stiffening its hindlimbs close to its body, and lifting its forelimb to alongside the tympanum.

**Distribution.** *Nyctixalus margaritifer* is distributed in highland areas above 700 m a.s.l. This species is currently known from the following areas:

- West Java: Gunung Gede near Tjibodas (Barbour 1912), Gunung Gede Pangrango National Park (Iskandar 1998), Gunung Halimun Salak National Park (MZB specimens HK 241/10.917 and 13/4030; Mumpuni 2001; Kurniati 2005), Bandung and Lebak Regencies (IUCN SSC Amphibian Specialist Group 2018), Telaga Warna Nature Reserve Bogor (Setiawan et al. 2019), and Sukabumi (MZB specimens RMB 2775/6749, RMB 2419/6686, and JAM 3130/6751).
- Central Java: Gunung Ungaran (MZB specimen U 43/16393), Purbalingga City (this study, NK 2118-19), and Banyumas Regency (this study, NK 2123-24)
- East Java: Sigogor Nature Reserve of Gunung Wilis (Dubois 1981; Priambodo et al. 2021) and Bromo Tengger Semeru National Park (this study, NK 2114 and NK 2122).

The distribution of *N. margaritifer* in Java is shown in Figure 6.

## Discussion

In this study, we provide comprehensive information on the current distribution, habitat, and description of *Nyctixalus margaritifer*, based on our newly collected specimens from BTSNP (East Java), and Banyumas Regency also Purbalingga City (Central Java). Our morphological analysis indicated that populations from East Java differ from those from West Java (Fig. 4). However, this was not confirmed by the genetic distance (Table 2) and phylogenetic results (Fig. 3), which failed to demonstrate a divergence and distinction between populations. The genetic distance threshold in the sister genus of *Nyctixalus*, *Theloderma* Tschudi, 1838, showed that the 3% interspecific 16S rRNA divergence, as commonly accepted in determining candidates for new species

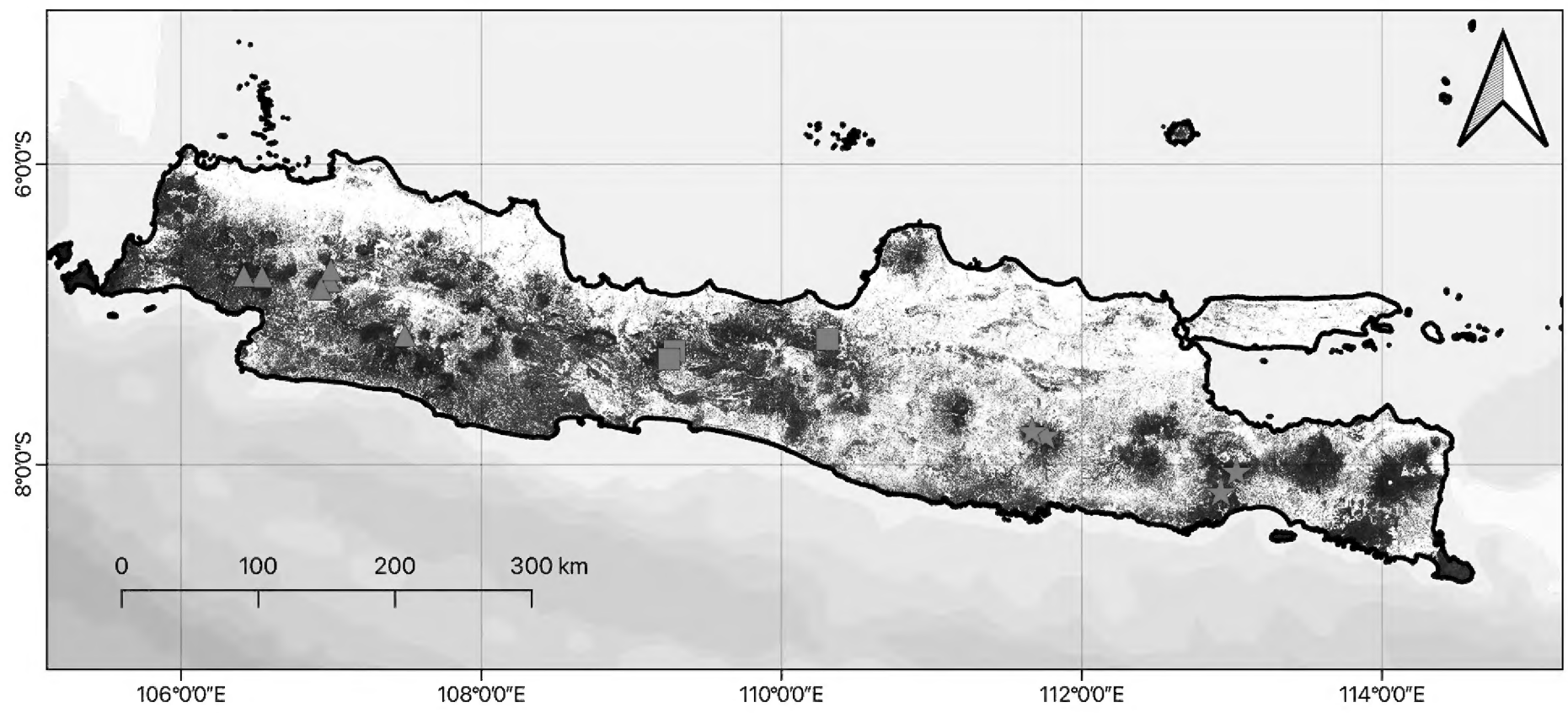


**Figure 6.** Geographic distribution of *Nyctixalus margaritifer* in Java showing type locality in Gunung Willis of East Java (red star), newly collected specimens from Bromo Tengger Semeru National Park of East Java (yellow star), newly collected specimens from Purbalingga City and Banyumas Regency of Central Java (yellow square), and other localities on Central Java (orange square) and West Java (orange triangle). See text (Distribution) for more information. Green area indicates tree cover in percentage (Hansen et al. 2013).

(Poyarkov et al. 2015; Kurniawan et al. 2023), was not met by our results. Additional samples are needed to resolve the taxonomic position of Javanese populations of *N. margaritifer.*

The distribution, ecology, and systematics of *N. margaritifer* are poorly understood. In East Java, there was only one specimen recently collected from the type locality on Gunung Willis (Priambodo et al. 2021), and with our additional specimens from BTSNP (NK 2114 and NK 2122), the species' geographic range now extends to easternmost Java, 142 km west of the type locality. Priambodo et al. (2021) briefly discussed notes on ecology and habitat of this species at other localities, and our findings are consistent; this species inhabits undisturbed rainforest to an elevation of 1,200 m a.s.l. and with a dense vegetation and canopy. It is possible that further localities in East Java will be found—possible such places could be Gunung Arjuno, Gunung Kawi, Gunung Argopuro, Ijen Crater Nature Park, Meru Betiri National Park, Alas Purwo National Park, and Baluran National Park—but the region must be thoroughly surveyed. *Nyctixalus margaritifer* has a relatively wide geographic distribution and presumably large population, and it has been listed as Least Concern by the IUCN Amphibian Specialist Group (2018), there is limited information on its occurrence, biology, systematics, and ecology; this hinders us in assessing possible threats, population trends, and the current conservation status of the species.

Although *N. margaritifer* is distributed across Java—with more reports in West Java than in East Java—the current habitat suited for this elusive species is, perhaps, in threatened, particularly in East Java. The highland forests in Java have decreased dramatically (Kurniawan et al. 2021), resulting in only tiny remnants and preserved forest. An analysis of habitat suitability modeling is needed to provide an overview of currently available habitat for *N. margaritifer.* There is also a gap in data coverage of herpetofauna diversity, especially in East Java, where only a few surveys have been conducted (Kurniawan et al. 2021; Kusrini et al. 2021). It can be assumed that the herpetofauna of East Java remains largely unknown, limiting us in determining appropriate conservation measures. Exploration and survey efforts will surely reveal many more distribution records or undescribed species. This has been proven by recent research which suggests that East Java may have a greater diversity of herpetofauna than is currently known; examples include new records of *Leptophryne borbonica* (Tschudi, 1838) (Erfanda et al. 2019), potential rediscovery of *Tetralepis fruhstorferi* Boettger, 1892 from Bromo Tengger Semeru mountain (Septiadi et al. 2023), and *Psammophis indochinensis* Smith, 1943 from Baluran National Park (Septiadi et al. 2022). In the absence of proper communication and overall database, it is also difficult to retrieve data on specimens kept in local university and museum collections. Therefore, synergistic fieldwork and collection-based studies are needed to uncover the hidden diversity of East Java.

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Fatansyah, K. Rimakhusshofa, M.W. Nauval, A.P. Baihaqi, B. Syamsiyah, and Dzulqornain; we also acknowledge NK Research members of Universitas Brawijaya, Indonesia, especially M. Farid and M. Asyraf R. We thank A.M.S.P. Soeprijadi for providing photographs of the habitats. We thank Amir Hamidy, Museum Zoologicum Bogoriense, for allowing us to examine their housed specimens. We are especially grateful to the anonymous reviewers for their reviews and helpful comments to improve the manuscript. We thank IDEAWILD for providing the equipment used in the study. This study was financially supported by *Litapdimas* of *Kementerian Agama Republik Indonesia* Research Grant (registration no: 221140000059527 to Berry F. Hanifa; registration no: 221140000059528 to Muhammad A. Hasyim), and the Rufford Small Grant for Nature Conservation (project ID: 36547-1) to Luhur Septiadi.

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Conceptualization: BFH, LS, NK. Data curation: MF, MPE. Funding acquisition: BFH, MAH, LS. Investigation: KP, MPE. Methodology: LS, KP. Resources: MR, HW. Supervision: MR, HW, NK. Project administration: MR, HW. Software: KP. Validation: MF. Visualization: LS, MF. Writing – original draft: BFH, LS, MPE. Writing – review and editing: LS, MAH, NK.

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# Appendix

**Table A1.** Detailed information on specimens of *Nyctixalus margaritifer* in Java, used for this study. NK: Laboratory of Animal Diversity and Ecology Collection, Biology Department, Universitas Brawijaya, Indonesia; MZB: Museum Zoologicum Bogoriense, BRIN-Cibinong, Indonesia; BP UM: Laboratory of Structure and Animal Development, Department of Biology, Universitas Negeri Malang, Indonesia

| Sex | Voucher ID | Locality | Depository | Observation | Sequenced (*n* = 2) | PCA analysis (*n* = 7) | Webbing formula (*n* = 11) | Sex comparison (*n* = 10) |
|---|---|---|---|---|---|---|---|---|
| Female | NK 2114 | Resort PTN Senduro, Bromo Tengger Semeru National Park, East Java | NK | Direct | ✓ | ✓ | ✓ | ✓ |
| Female | NK 2122 | Resort PTN Darungan, Bromo Tengger Semeru National Park, East Java | NK | Direct | ✓ | ✓ | ✓ | ✓ |
| Female | BP UM 0011 | Sigogor Nature Reserve, Mt. Willis, East Java | BP UM | Literature | X | ✓ | ✓ | X |
| Female | NK 2118 | Serang Pratin Village, Purbalingga City, Central Java | NK | Direct | X | X | ✓ | ✓ |
| Female | NK 2119 | Serang Pratin Village, Purbalingga City, Central Java | NK | Direct | X | X | ✓ | ✓ |
| Male | NK 2123 | Limpakuwus Village, Purwokerto District, Banyumas Regency, Central Java | NK | Direct | X | X | ✓ | ✓ |
| Male | NK 2124 | Limpakuwus Village, Purwokerto District, Banyumas Regency, Central Java | NK | Direct | X | X | ✓ | ✓ |
| Female | RMB 2775/6749 | Gede Pangrango Village, Kedungdampit, Sukabumi, West Java | MZB | Direct | X | ✓ | ✓ | ✓ |
| Female | HK 241/10.917 | Cibunar, Gunung Halimun National Park, West Java | MZB | Direct | X | ✓ | ✓ | ✓ |
| Female | RMB 2419/6686 | Gede Pangrango Village, Kedungdampit, Sukabumi, West Java | MZB | Direct | X | ✓ | ✓ | ✓ |
| Female | JAM 3130/6751 | Mt. Gede Pangrango, Kedungdampit District, Sukabumi, West Java | MZB | Direct | X | ✓ | ✓ | ✓ |

**Table A2.** Summary statistics and loading for the PCA analysis of *Nyctixalus margaritifer* from West Java and East Java populations, using 14 allometric body-size correction characters incorporating BP UM 0011 measurements by Priambodo et al. (2021).

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 |
|---|---|---|---|---|---|---|---|
| Standard deviation | 2.58 | 1.91 | 1.53 | 0.97 | 0.64 | 0.00 | 0.00 |
| Proportion of variance | 0.47 | 0.26 | 0.17 | 0.07 | 0.03 | 0.00 | 0.00 |
| Cumulative proportion | 0.47 | 0.74 | 0.90 | 0.97 | 1.00 | 1.00 | 1.00 |
| Eigenvalue | 6.64 | 3.66 | 2.35 | 0.93 | 0.41 | 0.00 | 0.00 |
| LAL | 0.38 | 0.10 | −0.04 | 0.03 | 0.22 | 0.00 | 0.17 |
| THIGHL | 0.36 | −0.11 | −0.18 | 0.05 | −0.22 | 0.12 | −0.28 |
| FOL | 0.34 | 0.00 | −0.03 | 0.08 | −0.72 | 0.06 | −0.31 |
| EL | 0.33 | −0.22 | −0.22 | 0.03 | 0.04 | 0.30 | 0.25 |
| IN | 0.28 | 0.23 | −0.34 | 0.02 | 0.17 | 0.17 | 0.19 |
| FTB | 0.21 | 0.43 | 0.04 | 0.03 | −0.05 | 0.10 | 0.26 |
| TL | 0.18 | 0.44 | 0.16 | 0.02 | 0.02 | −0.44 | −0.20 |
| TD | −0.05 | −0.29 | −0.53 | −0.01 | −0.14 | −0.69 | 0.16 |
| SVL | −0.11 | 0.00 | −0.01 | 0.99 | 0.06 | 0.00 | 0.00 |
| HAL | −0.12 | 0.48 | −0.15 | −0.02 | 0.11 | −0.13 | −0.34 |
| IOD | −0.13 | 0.06 | −0.60 | −0.04 | 0.27 | 0.25 | −0.46 |
| HW | −0.16 | 0.40 | −0.29 | 0.00 | −0.35 | 0.00 | 0.45 |
| HL | −0.37 | 0.11 | 0.02 | −0.02 | −0.31 | 0.25 | −0.09 |
| SL | −0.37 | 0.03 | −0.14 | −0.03 | −0.17 | 0.21 | 0.17 |